AN EVALUATION OF QUANTITATIVE METHODS FOR MEASURING COLD HARDINESS OF CITRUS SEEDLINGS

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I. INTRODUCTION

Plant life is influenced to a great extent by the interactions of a variety of environmental factors. One of the most important environmental factors is climate, which determines largely the types of plants grown in a particular locality and brings about profound modifications in their normal rates of growth and other physiological responses. Climate also limits the extent in range of plant species as definitely as would a physical barrier. Among the various elements of climate, temperature plays a major role in the growth of plants. All physiological processes within the plant, such as photosynthesis, respiration and growth, are directly or indirectly influenced by temperature.

With the many processes involved it is not surprising that the plants belonging to different genera, species and varieties exhibit high degrees of diversity in their adaptations to various ranges of temperature. For example, many of the deciduous tree fruits are limited to temperate regions with sufficient low temperatures to break their rest periods, while the evergreens of the subtropics, such as the citrus fruits and mango, can not withstand severe freezes and require protection during cold periods.

The problem of low-temperature injury to tender subtropical plants is of great importance in parts of the world where considerable diversities of temperature exist. Freezes resulting from sudden drops in temperature present a continuous hazard to successful growing of such crops. The situation in some of the areas is further aggravated by alternating periods of high and low temperatures. Such extremes of temperature make cold-susceptible fruit trees particularly prone to extreme damage and eliminate large expanses of otherwise suitable land from use for growing them.

Citrus production is greatly influenced by low temperatures in winter. Whereas relatively low winter temperatures are conducive to an excellent internal and external fruit quality, temperatures below freezing often prove to be limiting factors in successful growing of citrus.

Temperatures below freezing have ravaged the citrus plantings of certain countries of the world to varying degrees. Most citrus growing regions of the United States have, at one time or other, been visited by severe low temperatures causing great damages to the citrus plantings of the respective areas. The citrus industry of Florida has received many setbacks from severe freezes and as a result citrus production has been concentrated in the central and southern parts of the state.

Temperatures below freezing are of common occurrence in Florida. Meteorological records show temperatures of 32°F. as early as October 24 and as late as April 17. There is no point on the mainland of Florida that has not been visited by frost at one time or other. Under such circumstances it becomes highly important to find ways to minimize the hazard due to cold temperatures beyond what may be done through selection of relatively frost-free sites and good production programs.

One solution of this problem may be sought by initiating a breeding project for developing cold-hardy citrus varieties. Another may arise through a program of physiological pre-conditioning of plants which may enable them to withstand sub-freezing temperatures without injury. A precise knowledge of the degree of cold tolerance and detailed data concerning responses of a species or variety to freezes under specified environmental conditions would be of great importance. These would be helpful in understanding the basic physiology regarding the cold tolerance of plants. Also, such information would be of significance and usefulness to the plant breeder who may thus be able to incorporate factors of frost resistance into his breeding program.

During the investigations reported herein, quantitative methods for the measurement of cold hardiness of plants were evaluated with a view to finding a reliable method suitable for citrus seedlings. The experiments comprising this study were conducted under controlled climatic conditions and may not necessarily agree with conceptions based on behaviour of plants under field conditions. It is hoped, however, that the knowledge gained from this study may prove beneficial in citrus breeding investigations and to those concerned with the pre-conditioning of plants. The actual freezing test, which was found to be the only reliable method to measure the cold hardiness of citrus seedlings, may also be helpful to the citrus industry through the elimination of non-hardy plants in the early stages of development.

II. REVIEW OF LITERATURE

A. General

From time immemorial man in collaboration with Nature has been making selections of plants which could thrive in a particular environment. It is natural that the cold hardiness of plants has received considerable attention from the investigators in the past. During the last few centuries many concerted efforts have been made to investigate and unravel the mysteries of cold hardiness of plants (8). The results of researches regarding the fundamental nature of cold hardiness of plants have been reviewed by many able investigators (8, 40, 48, 65, 75).

B. Methods of Measuring Cold Hardiness

Along with the research dealing with the physiology of cold hardiness, attempts have been made to find practical methods of measuring the cold hardiness of plants. The availability of such a reliable measure of hardiness is highly desirable for physiological or chemical investigations of the problem, since the degree of hardiness of the material one is working with should be known to reach any valid conclusions (69).

A survey of literature dealing with cold hardiness showed that many methods, simple as well as ingenious, have been employed by various investigators to measure the cold hardiness of plants. For a simpler treatment of the subject, these methods can be classified into four broad categories.

1. Field-survival method

This method seems to be the oldest of all the methods for measuring the cold hardiness of plants. Since ancient times the cold tolerance of plants has been measured by the ability of plants to survive in the field. In this method, plants are grown in the field under ordinary growing, hardening and freezing conditions. The injury to plants is determined by bringing samples into the greenhouse at specific intervals or by examining them in the spring. Test winters, winters of cold weather of an average severity, are essential for such a test to be locally reliable (15). Generally the test winters occur about every ten years (87).

The field-survival method has been used by many workers for measuring the cold hardiness of red raspberries (2), winter oats (11), parsnip (14), tung (18, 58, 68), flax (19), small grains (28), subtropical and tropical plants (31) and apples (51).

The field-survival method has been successfully used by Coffman to measure the cold hardiness of oats in a breeding program (11). Each entry in the nursery was sown in duplicate and estimates of the stand of winter oat plants were made in the fall after the plants had emerged and again in the spring after danger from winter injury had passed. The results obtained by calculating the survival percentages of different varieties were used in extending the cultivation of winter oats further northward.

Cold tolerance of flax varieties sown in fall was tested by Dillman (19) during three winters by the field-survival method and selections were made from the surviving plants for use in further breeding. Hodgson et al. (31) in California used this method for estimating the degree of cold hardiness of citrus varieties and many other subtropical and tropical plants.

The field-survival method, although accurate, is a slow method and a true picture of the cold hardiness of a plant may not at times be provided due to the interactions of the many complex environmental factors in the field (40). Furthermore, since the subnormal cold periods do not usually occur twice under similar climatic conditions, the conclusions drawn from such comparisons may not always be valid.

2. Plant anatomy and morphology methods

Attempts have been made by many observers to correlate the morphological characters of plants with their cold hardiness (19, 79). Working with flax, Dillman (19) tried to correlate the branched habit of growth with the cold hardiness of these plants. Beach and Allen (3) working with apples found some indications of morphological differences between hardy and tender varieties; yet from the practical point of view it was impossible for them to name any one character by which the degree of constitutional hardiness of a seedling apple may be foretold. Among the various characters related to cold hardiness, they suggest, that of the length of season's growth is of first importance. They noticed that the hardier varieties of apples mature their wood earlier. It seems, however, that under natural conditions when the tree is subjected to a multitude of environmental factors, this character could not be relied upon as a suitable method for measuring the cold hardiness of apples.

Investigations have also been carried on to find out the possibility of any correlation between the anatomical characteristics of plants and their cold hardiness (7, 13, 32, 41, 60, 78). Sakai (60) found that in the mulberry tree there was a parallel correlation between the length of the corkless part of twigs in early autumn and that of parts which died of cold temperatures in winter. According to him the estimation of the length of the corkless part of twigs in early autumn is a practical measure of their cold hardiness. Cooper et al. (13) found that the cambial activity of citrus plants influences their cold hardiness. They reported that the treatments which induce dormancy of cambium increase cold hardiness of the citrus plants.

The relationship between the cell size and cold hardiness of plants has received considerable attention. Because of the greater specific surface and more nearly spherical shape, the small cell size is considered to reduce the danger of cold injury. Reduced water content and increased sugar content would both insure a less severe cell collapse, the former because less water is available to be removed, the latter because of osmotic forces opposing water removal (39). In this connection, Chandler (7) working with a number of plants found that the small cell size had a relationship with the increased cold hardiness of the plants. Levitt and Scarth (41) also reported that the small cell size was characteristic of hardy plants.

A positive relation between small cell size and the cold hardiness of plants has been found by many investigators (40). Review of literature on this subject, however, showed that no work has been done to find a correlation between the small cell size and the cold hardiness of citrus plants. Mendel believes such a correlation to be highly doubtful.[1] There are even many examples of hardier plants having larger cells (40).

During the investigations of Levitt, Scarth and Siminovitch (41, 42, 64, 65, 74, 75) much light was shed on the physical properties of protoplasm in relation to cold hardiness. These workers found that the protoplasmic viscosity decreases on hardening of plants. In this state of resistance to cold injury, the protoplasm shows a marked ability to remain ductile and to recover easily when subjected to profound dehydrating forces. Working with mulberry Sakai (61) found, with some minor discrepancies, higher permeability to water in the case of hardy cells.

Briggs and Siminovitch (4, 69) also made use of the viscous property of the protoplasm and reported it as a reliable measure of cold hardiness of the black locust tree. Their later work (70) thoroughly examined and established the validity of the use of plasmolysis and desiccation tests for estimating the cold hardiness of the bark tissue of the black locust tree.

3. Chemical methods

During the course of investigations to find the nature and cause of cold hardiness, many workers tried to link some chemical constituent of plant tissue with its degree of cold tolerance. Maximov (48) considered the protective role of soluble sugars and other closely allied substances in frost endurance to be quite indubitable. He further affirmed that although sugars were not the only possible protective substances, nevertheless, it was mainly these compounds from which the plants acquire cold hardiness under natural condition. Kneen and Blish (37), working with winter hardiness of wheat, presented data showing that winter wheat varieties which displayed a high degree of cold hardiness in the hardened condition also proved capable of building up higher dry matter and sucrose contents than varieties of lesser cold resistance and winter hardiness.

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[1] Kurt Mendel. 1958. Personal communication.

Although sugars may be very important for their role in cold hardiness of certain plants, in many cases this relation does not hold true. Many of the hardiest of the evergreens, especially among the conifers, show little parallelism between hardiness and sugar content (39). Ivanov (32, 33, 34) in his work with citrus species could not detect any correlation between frost resistance and the sugar content of the plants. Siminovitch and Briggs (71, 72) presented evidence indicating that variations in soluble sugars in the bark tissue of the black locust bear no relationship to the frost hardiness of the cells.

Some of the attempts to investigate the relationship between nitrogenous substances, particularly proteins, and cold hardiness of plants were successful. Strausbaugh (79) found evidence to indicate that there was a modification in the protein of the buds in dormant plum trees. Loomis (43) observed that large quantities of protein nitrogen were stored in winter in the bark and wood of trees. The work of these and many other earlier investigators was of little value in determining some relationship between nitrogenous substances and frost hardiness, primarily because the methods used were too crude to obtain results of reliable precision (40).

The development of newer biochemical techniques in recent years encouraged scientists to explore further the relationship between nitrogenous substances and cold hardiness of plants. Briggs and Siminovitch (4, 69, 71) were pioneers in studying the chemistry of the living bark in relation to cold hardiness. Earlier Jones *et al*. (35) had shown the possibility of some relationship between protein content and cold hardiness of the black locust trees.

Siminovitch and Briggs (69) studied the changes which occur seasonally in the water-soluble proteins, the water-insoluble proteins, and the water-soluble non-protein nitrogen of the living bark tissue of the black locust in relation to the seasonal variations in its cold hardiness. Only the water-soluble proteins were found to increase with hardening during the fall and decrease with hardiness in the spring. Their subsequent work (4, 71) confirmed the existence of a direct correlation between the water-soluble protein content of the cells and their ability to withstand injury by extracellular freezing or by dehydration. Mirimanjan (50) also found higher water-soluble proteins in hardy plants than in the cold-susceptible plants.

Chandler (8) considers that the water-soluble protein, through its modifying influence on the physical properties of protoplasm increases the cold hardiness of plants. According to Levitt (39), on the other hand, the significance of this protein in cold hardiness cannot yet be explained.

4. Electrical conductivity methods

Dexter and co-workers (16, 17) developed a method of measuring the cold hardiness of plants on a quantitative basis. This method consists of exposing the plant or a plant part to low temperatures. The degree of resistance of the plant to injury by low temperature is then measured by the diffusion of electrolytes and other substances from chilled or frozen tissues into water after such tissues have thawed. This method is based on the theory that exosmosis of electrolytes from frozen plant tissue is proportional to the amount of freezing injury.

Dexter et al. (16) took alfalfa roots of three varieties of known hardiness and froze them under controlled conditions for various lengths of time in a large thermostatically regulated electrical refrigerator. Some of the frozen roots from each lot were planted in the greenhouse, where their recovery and growth was observed. Distilled water was added to the tubes containing the remaining frozen roots. After ten hours electrical resistance readings were taken on the electrolytes obtained from the roots. The results of these experiments showed a correlation between known hardiness of alfalfa roots and the degree of release of electrolytes from the tissues after freezing.

In agreement with Dexter et al. (16, 17) many other workers have reported success with the use of this method. Cold hardiness has since been measured by this method for roses (6), grapefruit (12), alfalfa roots (16), clover and small grains (17), apples (21, 22, 26, 80, 81, 82), cherries (23), grapes (24), lemons, mandarins and oranges (25), mulberries (62), woody ornamentals (77) and some other plants of Malus species (88).

Scott and Cullinan (66) tried to detect the degree of cold injury to the fruit buds of peaches by the electrical conductivity method and found it unsatisfactory. However, they recognized its usefulness for measuring the cold hardiness of stems and roots of many other crops.

Campbell et al. (5) and Fillinger et al. (27) used a modified version of the conductivity method for measuring the cold hardiness of plants. They employed two pairs of electrician's pliers with needles attached to their jaws to make contact with the plant. A resistance measure was used to determine the cold hardiness of grape canes and crab apple seedlings respectively. This modified method of measuring the electrical conductivity seems to have many advantages such as saving of time and reduction in the variability due to sampling techniques as compared with the original method developed by Dexter.

C. Treatment of Plant Material

1. Controlled temperature rooms

Realizing that the field-survival method of measuring cold hardiness of plants was very slow and affected by many environmental factors, certain investigators developed means of artifically freezing the plant tissues under standard conditions and then making an estimate of injury. The pioneer in artificial freezing tests was probably

Chandler (7) who used a small freezing chamber to freeze the plant tissues. The temperature was lowered by the use of mixtures of ice and salt. A similar but more elaborate freezing chamber was developed by Potter (57).

Hildreth employed a mechanical refrigeration plant to control temperatures in the freezing chambers (30). Many workers have used deep-freeze units (9, 20, 59) or some other modifications of the freezing chambers (1, 10, 46, 67) for testing the cold tolerance of plants.

Generally, very good agreement between the controlled freezing tests and field-survival values has been reported by Hildreth (30), Kinbacher (36), Kneen and Blish (37), Martin (45) and Weibel and Quisenberry (84).

Earlier experiences and the ingenuity and imagination of investigators culminated in the construction of air-conditioned greenhouses (85, 86) in which the control of environmental factors is achieved to a great degree. The use of greenhouses of this type, besides yielding purely practical information such as the degree of cold resistance of different varieties and species of crop plants, puts into the hands of investigators a powerful means for further study of the problem of physiological factors (48).

2. Hardening and freezing of plants

When the plants are treated in the artificial freezing chambers, a standard procedure with respect to condition of plants and rate of fall and duration of low temperature has to be adopted for valid comparisons.

Although no single temperature has been used by different workers for hardening of plants, most of them agree that the plants should be hardened at above-freezing temperatures for about one week (1, 33, 76). Reports indicate that the threshold temperature for hardening of plants seems to be about 43°F.

The rate of fall of temperature is very important in the cold hardiness studies. Some of the workers used arbitrary values of 1.7°F. (63) or 4°F. (9) per hour. Many other investigators used intermediate rates of fall of temperature (22, 24, 26). Similarly the duration of low temperature to which the plants were subjected for freezing treatment varied from three hours (12, 13, 30) to 12 hours (45).

3. Estimation of injury to plants

When the plants or plant tissues are subjected to freezing temperatures in Nature or in the freezing chambers, the degree of cold injury received by them can be estimated by the following methods.

A. Visual method: The visual method of estimating injury consists in observing the plant tissue for color and appearance. A brownish coloration and/or water-soaked appearance of tissue indicates that it is dead. This method has frequently been used for peaches (7, 9, 20, 38, 63, 66), grapefruit (12), apples (21, 25), grapes (24), black currants (52) and woody ornamentals (77).

B. Greenhouse-recovery method: Plants which have been subjected to cold temperatures are brought into the greenhouse and are allowed to remain there for a few days. They are then scored for degree of recovery. Swingle (81) made a careful check of the exosmosis method against this "freeze and wait" method of determining the injury. He found complete agreement between the results obtained by electrical conductivity readings and the observations made on the material put in the greenhouse at 60°F. for seven days. Angelo (1), Clark (10), Head (29) and Sjoseth (76) also used this method with success.

c. Vital staining: The technique of differential staining has been used by many scientists to differentiate between living and dead plant cells. Mattson _et al_. (47) and Parker (54, 55) used triphenyltetrazolium chloride to determine the viability of plant cells. Carrier (6) reported, however, that when the methods of determining the extent of freezing injury to roses were compared, the ability of the tissues to convert the tetrazolium chloride to red color was the least satisfactory means of estimating the ability of a tissue to survive freeze injury.

Luyet (44) described the technique of using neutral red to test the viability of plant cells. The living cells acquire a bright cerise color with the use of this stain, and the dead cells, on the other hand, are of an intense orange-yellow. The contrasts, observed Luyet, were violent.

Neutral red is a vital stain and is a great aid to the superficial observational methods (53). Siminovitch and Briggs (70) also used this stain to differentiate between living and the dead cells.

III. MATERIALS AND METHODS

A. Controlled Climate Chambers

To provide optimum growing conditions which could be reproduced, special climate chambers were constructed for the growing of plants. These consisted of two growth chambers for growing plants. A cold chamber was also provided for subjecting the plants to desired low temperatures. The growth chambers, which were 8' x 8' x 7 1/2' in dimension, were made from walk-in type refrigerators in which the desired temperature could be maintained (Fig. 1). The double-walled chambers were constructed of tongue-and-groove fir and were well insulated with rock wool ranging from three to six inches in thickness, depending upon the minimum operating temperature requirements. Cooling was obtained by the use of evaporative coils mounted on opposite inside walls. Air-cooled condensing units were installed to obtain the desired temperature. Lighting for the growth chambers was provided by F72 T12OW "Slimline" fluorescent lamps, spaced about two and three quarter inches apart, mounted in the ceiling. To provide satisfactory light quality six 100-watt incandescent lamps were also used in the growth chambers. An interior view of one of the growth chambers is shown in Fig. 2.

B. Plant Materials

The research reported in this study was conducted using seedlings of three species of Citrus -- C. aurantifolia Swingle, C. limon Linn. and C. aurantium Linn. These species were chosen because they were believed to present a wide range in their relative degree of cold

Figure 1. Controlled climate chambers.

Figure 2. Interior view of one of the growth chambers.

hardiness under natural conditions. Citrus aurantifolia is considered to be less cold hardy than the other two species, C. limon is medium, and C. aurantium is the most cold hardy of the three species under study (83). This knowledge was considered to be helpful in the evaluation of methods for measuring the cold hardiness of the seedlings.

Seeds of Key lime (C. aurantifolia), Rough lemon (C. limon) and sour orange (C. aurantium) were sown in greenhouse flats containing a mixture of No. 5 crushed granite rock and vermiculite in equal volumes as the rooting medium. All the seedlings were grown under similar conditions of temperature, light and humidity. A standard nutrient solution (modified Hoagland solution (86)) was used throughout the experiments.

A mild and scattered attack of anthracnose (Colletotrichum gloeosporioides Penz.) was noticed on the young leaves especially of Key lime and Rough lemon seedlings. Ferbam, one pound per 100 gallons of water, was sprayed on all the seedlings. This treatment was repeated at weekly intervals till the disease was controlled.

When the seedlings were about six months old, they were potted in four-inch plastic pots containing the same rooting medium which was used in the greenhouse flats. Care was taken to select seedlings of approximately uniform height and vigor within each species.

The potted seedlings of the three species of citrus were then placed in one of the growth chambers. The temperature in this chamber was maintained at 78°F. A 14-hour daily illumination period was provided throughout the growing and hardening stage of the seedlings.

The intensity of light, three feet from the lamps (near the tops of the seedlings) was about 1600 foot-candles. The seedlings were subterraneously supplied with modified Hoagland nutrient solution, diluted with an equal volume of water, once each day by means of an automatic pumping system. After the pumps stopped, the nutrient solution drained back into the stainless-steel tanks. The solution in these tanks was changed every two weeks.

For the first series of experiments, 54 seedlings each of Key lime, Rough lemon and sour orange were thus grown in the growth chamber at 78°F. After four weeks the seedlings were randomly divided into six equal groups, each having nine seedlings of each of the three species. These groups of seedlings were treated as follows.

Group A1 was taken to the laboratory for the electrical resistance measurements (Table 1).

Group B1 was analysed for water-soluble proteins (Table 6).

Groups C1 and D1 were hardened at 38°F. for two weeks.[1] The seedlings of group C1 were used for taking the electrical resistance readings (Table 1) and those of group D1 were analysed for water-soluble proteins (Table 6).

Groups E1 and F1 were hardened at 38°F. for two weeks along with groups C1 and D1. The seedlings of groups E1 and F1 were then placed in the cold chamber at 35°F. After the seedlings remained at 35°F. for three hours, the temperature in the cold chamber was lowered to 23°F. at the rate of 2°F. per hour. The temperature was maintained at 23°F. for three hours and then raised to 35°F. at the rate of 2°F. per hour. The temperature inside the cold chamber was recorded with the use of thermocouples and a recording potentiometer. When the seedlings had thawed at 35°F., those of group F1 were placed in the growth chamber at 78°F. to observe their recovery. The seedlings of group E1 were taken to the laboratory for making resistance readings.

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[1] The term "hardening" as used hereafter refers to seedlings which have been exposed for a period to temperatures of 50°F. or below, and does not necessarily indicate that any greater resistance to cold has been induced.

For the second series of experiments, 64 seedlings of each of the three species of citrus were grown in one of the growth chambers at 78°F. After four weeks the seedlings were randomly divided into eight equal groups, each having eight seedlings of each of the three species. These groups were handled according to the following schedule.

Group A2 was taken to the laboratory for taking electrical resistance readings on the seedlings (Table 2).

Group B2 was analysed for water-soluble proteins (Table 7).

Groups C2 and D2 were placed in another growth chamber at 78°F.

Groups E2, F2, G2 and H2 were hardened at 38°F. for ten days. After they were hardened for ten days at 38°F., the seedlings of group E2 were taken to the laboratory for measuring their electrical resistance (Table 2) and those of group F2 were analysed for water-soluble proteins (Table 7). The seedlings of groups G2 and H2 along with the seedlings of the groups C2 and D2 were placed in the cold chamber at 75°F. The temperature in this chamber was brought to

35°F. at the rate of fall of 10°F. per three hours. After the plants remained at 35°F. for three hours, the temperature in the cold chamber was lowered to 23°F. at the rate of 2°F. per hour. The temperature was maintained at 23°F. for three hours and then raised to 35°F. at the rate of 2°F. per hour. A continuous record of the temperature inside the cold chamber was obtained using the method previously described.

When the seedlings had thawed, those in groups C2 and G2 were taken to the laboratory for measuring their electrical resistance (Table 2). The seedlings of groups D2 and H2 were placed in the growth chamber at 78°F. for three weeks to observe their recovery (Table 9).

C. Methods Used for Measuring Cold Hardiness

The following methods for measuring the cold hardiness of citrus seedlings were evaluated during this study.

1. Electrical conductivity of tissue extract

The choice and the treatment of plant tissues for the electrical conductivity experiments were guided by the exploratory work carried out on the seedlings. Of the tissue obtained from various parts of the seedlings, the leaves and the stem gave fairly consistent resistance readings. However, leaves were preferred over the stem tissue because if stem was to be used in the experiments, the whole seedling had to be cut into pieces. The use of the stem, therefore, was not considered desirable since its use resulted in the loss of the plant itself.

Before taking the samples, all the leaves of the seedlings were thoroughly cleaned with a dry cheese cloth. For the first series of experiments all the leaves of the seedlings were removed and weighed promptly. The leaves were then cut into one-quarter inch strips and put in an Erlenmeyer flask. Distilled water was added to each flask at the rate of exactly ten times the weight of the tissue and the flasks were stoppered. For the second series of experiments, five grams of leaves were taken from each seedling. Approximately one-third of these leaves were contributed by each of the top, middle and the lower parts of the seedlings respectively. The leaves were cut into one-quarter inch strips and put into a 125 ml Erlenmeyer flask. Twenty-five ml of distilled water was added to each flask and the flask was then stoppered.

The stoppered flasks, in both first and the second series of experiments, were allowed to stand for 24 hours at 78°F., after which time the electrical resistance readings of the water extract from the leaves were taken at 78°F. A type R C conductivity bridge was used for this purpose.

2. Electrical conductivity of intact tissue

A special device was constructed in this laboratory to measure the electrical resistance of the intact seedlings. It consisted of two sharp tungsten needles set parallel in a Pyrex glass block. In the first series of experiments the needles were spaced 2 in. apart. For the second series of experiments the needles were set exactly 1 cm. apart. The later setting was selected in order to make the spacing comparable with that of the platinum electrodes used to measure the conductivity of the tissue extract.

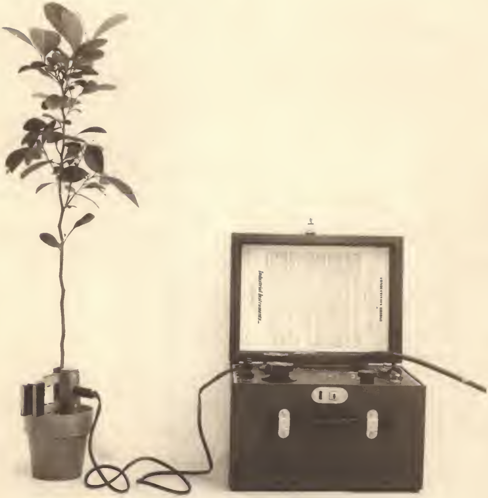
The tungsten needles fixed in the glass block were fastened to a wooden jaw in such a way that about a 3 mm. length of the sharp tip end of each needle was free to act as a probe. A spring was attached to the jaw so that the instrument could be attached to the seedling firmly when the needles were inserted in it. Figure 3 shows in detail the construction of this device.

Leads from the needles were attached to a type R C conductivity bridge. In the first series of experiments, the electrical resistance was measured at the top, near the second node from the apex of each seedling. In the second set of experiments, the resistance was measured at three points on each seedling: (1) at the base of the seedling just above the rooting medium, (2) at the middle, near the junction of the current and the last season's growth, and (3) at the top, near the second node from the apex of the seedling.

3. Estimation of water-soluble proteins

For the analysis of water-soluble proteins, the leaves and the bark tissue were thoroughly cleaned with a dry cheese cloth and removed from the seedlings. Each sample was weighed promptly, cut into small pieces, and was put into a Waring blendor jar. Sufficient distilled water at 35°F. was added and the tissue was then blended for two minutes. The blended tissue was allowed to stand over night at 35°F. and filtered, using Whatman #40 filter paper. The tissue remaining on the filter paper was dried at 105°C. for 24 hours for dry matter determination. The filtrate was used for estimation of water-soluble protein.

Figure 3. Instrument for measuring electrical resistance on intact seedlings.


Industrial Instruments

Trichloroacetic acid (100 percent solution) was added to the filtrate at 5 ml of acid per 100 ml of the extract. The extract was constantly stirred at the time of addition of acid. After 12 hours the precipitate was filtered off and thoroughly washed with hot acetone until a clear filtrate appeared.

The nitrogen content of the precipitate was determined by the macro-Kjeldahl method, and the results were expressed in terms of percent water-soluble proteins in the tissues sampled on a dry-weight basis.

4. Plasmolysis test

A piece of stem approximately one inch in length was taken from the portion of seedlings adjacent to the rooting medium. Tangential sections twenty-five microns in thickness were cut from the bark tissue of the stem pieces with a sliding microtome. Each section was then cut into three equal parts. These subsections were subjected to the dehydrating force of a balanced salt solution ($NaCl : CaCl_2 :: 9 : 1$) for ten minutes. During these tests, three concentrations of the balanced salt solutions were tried: 1M, 2.5M and 5M. Upon removing from the salt solutions, the subsections were placed in tap water for five minutes in order to deplasmolyze the cells. They were taken out of water and blotted quickly with filter paper before putting them in neutral red solution for a few minutes. After staining, the subsections were washed in distilled water for ten minutes and examined under the microscope. The cells which retained the stain were considered to be living and the cells which did not retain the stain were considered to be dead.

5. Actual freezing test

For the first series of experiments nine seedlings each of the three species comprising group F1 (page 22) were grown at 78°F. for four weeks. These seedlings were then hardened at 38°F. for two weeks before exposing them to freezing temperature. For the second set of experiments, groups D2 and H2 (page 23) each having eight randomly selected seedlings of Key lime, Rough lemon and sour orange were taken. The seedlings of both these latter groups were grown at 78°F. for four weeks in the growth chamber. After that time the seedlings of both the series 1 and 2 were subjected to hardening and freezing temperature treatments as outlined on pages 22 and 23 respectively.

Three weeks after the respective freezing temperature the seedlings were examined for their recovery and each seedling was rated as follows:

Index Description (illustrated in Fig. 4)

0 = Dead

1 = Heavy injury and slight recovery

2 = Medium injury and moderate recovery

3 = Slight injury but complete recovery

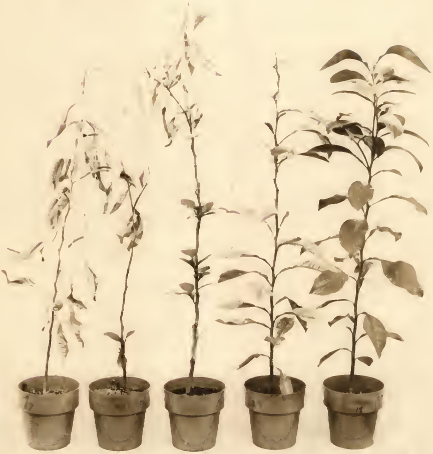
4 = No injury

The total percent recovery for each species was computed with the following formula:

$$\frac{25\,(\Sigma\ \text{Index})}{N} = \text{percent recovery}$$

Figure 4. The sour orange seedlings three weeks after cold treatment, showing the degrees of recovery in the growth chamber.

(L to R) Dead (0)
Heavy injury and slight recovery (1)
Medium injury and moderate recovery (2)
Slight injury but complete recovery (3)
No injury (4)



Where

25 = Recovery factor

N = Number of seedlings in each treatment

The working of the formula may be explained by the following example. In the first series of experiments, nine seedlings of Key lime were subjected to the freezing temperatures and subsequently their recovery indexes were noted. The sum of the nine indexes was 4 for that species. The total percent recovery, therefore, was $\frac{25 \times 4}{9} = 11.11$. The total percent recovery for the other species was calculated in the same manner.

IV. EXPERIMENTAL DATA AND RESULTS

The experimental data and results obtained during these studies are presented below.

A. Electrical Conductivity of Tissue Extract

1. First series of experiments

After the citrus seedlings had been given the respective temperature treatments, those of groups A1, C1 and E1 were used for electrical resistance measurements. The specific electrical resistances of the leaf extract of seedlings of the three species are presented in Table 1.

The data showed that the resistance readings for the Key lime seedlings were considerably higher than for Rough lemon and sour orange seedlings. This trend was manifest for all the temperature treatments, that is, the readings taken before freezing and after freezing of seedlings. The resistance readings for Rough lemon, however, were lower than those of sour orange. The electrical resistance of sour orange seedlings was considerably higher than that of Rough lemon seedlings in the treatment where readings were taken on the unhardened seedlings before subjecting them to freezing temperature. This difference, nevertheless, narrowed down greatly in the case of seedlings which were exposed to cold temperatures.

The data further showed that the resistance readings of seedlings which were exposed to freezing temperature were considerably higher than those for the seedlings which received the hardening treatment only.

TABLE 1

SPECIFIC ELECTRICAL RESISTANCE, EXPRESSED IN OHMS (x 10), OF LEAF EXTRACT OF THREE SPECIES OF CITRUS* USED IN THE FIRST SERIES OF EXPERIMENTS

Kind of seedlings	Before freezing		After freezing
	Unhardened	Hardened	Hardened
Key lime	792.6	191.9	230.7
Rough lemon	224.2	85.9	92.5
Sour orange	386.6	88.7	113.4

*All figures shown are averages of nine seedlings.

2. Second series of experiments

Eight seedlings of each of the three species of citrus were used for each experiment in this series. The electrical resistance readings were taken before and after these seedlings were subjected to freezing temperatures. The seedlings thus treated comprised groups A2 and C2 which were not exposed to 38°F., and groups E2 and G2 which had been hardened at 38°F. for ten days. The electrical resistance readings taken on the seedlings thus treated are presented in Table 2.

The resistance data showed that the electrical readings had no correlation with the corresponding percent recovery of the three species (Table 9). In the case where readings were taken after freezing, the unhardened Key lime seedlings showed considerably higher resistance than both Rough lemon and sour orange. The hardened Key lime seedlings also showed higher resistance readings as compared to Rough lemon. Readings taken on the seedlings which were not exposed to the freezing temperatures, on the other hand, showed that the unhardened Rough lemon had the highest resistance followed by sour orange and Key lime respectively. There was a marked decrease in resistance in the hardened seedlings. In this case Rough lemon seedlings had the lowest readings of the three species. The readings taken on the seedlings before and after freezing, however, showed that the electrical resistance decreased considerably in the hardened seedlings as compared with the unhardened seedlings.

TABLE 2

SPECIFIC ELECTRICAL RESISTANCE, EXPRESSED IN OHMS (x 10), OF LEAF EXTRACT OF THREE SPECIES OF CITRUS* USED IN THE SECOND SERIES OF EXPERIMENTS

Kind of seedlings	Before freezing		After freezing	
	Unhardened	Hardened	Unhardened	Hardened
Key lime	704.8	408.8	596.0	127.8
Rough lemon	1361.2	324.0	273.9	123.0
Sour orange	1141.9	537.2	476.4	170.6

*All figures shown are averages of eight seedlings.

The electrical resistance readings for the hardened and the other seedlings which were exposed to the freezing temperature were then expressed in percent decrease over the readings for the unhardened group which was not treated with cold temperatures. The data thus obtained are graphically presented in Fig. 5. The Key lime seedlings showed the least percent decrease (supposedly indicative of highest cold hardiness). The sour orange seedlings had the highest percent decrease in the resistance readings. These data are in marked contrast to the relative cold hardiness values of the three species obtained in the actual freezing test (Table 9).

B. Electrical Conductivity of Intact Tissue

1. First series of experiments

Groups A1, C1 and E1, each having nine seedlings each of Key lime, Rough lemon and sour orange, were used for taking the electrical resistance readings on the intact stem tissues. These seedlings were the same from which the leaves were used for measuring the electrical resistance of the tissue extract.

The average resistance readings for the intact main axes of seedlings of Key lime, Rough lemon and sour orange are presented in Table 3.

The data in Table 3 showed that the Key lime seedlings had the highest electrical resistance for all the temperature treatments. The readings for sour orange were higher than those for Rough lemon in the case of seedlings where the readings were taken before hardening and after freezing. In the case of hardened seedlings, the readings for Rough lemon were higher than those of sour orange.

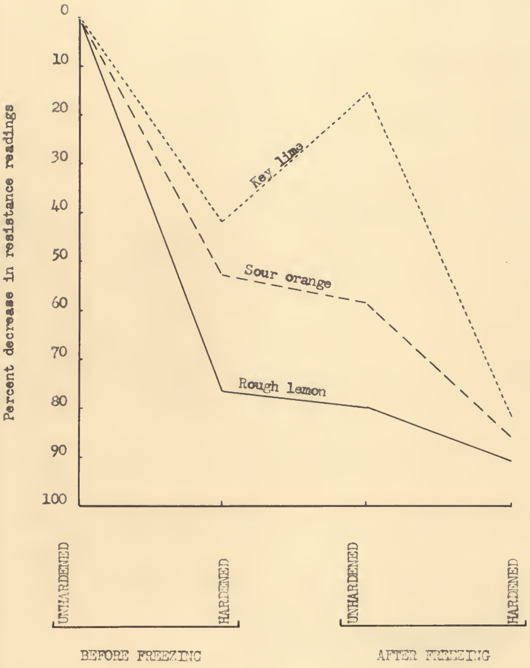


Fig. 5. Percent decrease in electrical resistance readings of citrus seedlings which had been exposed to cold temperatures over the seedlings grown at 78°F.

TABLE 3

THE AVERAGE RESISTANCE READINGS*, EXPRESSED IN OHMS ($\times 10^3$), TAKEN ON THE INTACT MAIN AXES OF SEEDLINGS OF KEY LIME, ROUGH LEMON AND SOUR ORANGE

Kind of seedlings	Before freezing		After freezing
	Unhardened	Hardened	Hardened
Key lime	541.7	434.0	234.4
Rough lemon	344.0	327.8	200.0
Sour orange	357.2	252.2	209.0

*These readings were taken by the probe with needles fixed 2 in. apart. Each entry in the Table is the average of readings taken on nine seedlings.

2. Second series of experiments

In the above experiments the electrical resistance readings were taken only near the apex of the seedlings. It was considered worthwhile to follow the response of the various parts of the seedling stems to different temperature treatments. This was done in the second series of experiments. Eight seedlings each of Key lime, Rough lemon and sour orange were used for each of the temperature treatments. The seedlings were the same which were used for measuring the electrical resistance of the leaf extracts and belonged to groups A2, E2, G2 and C2.

The average resistance readings for the intact seedlings of Key lime, Rough lemon and sour orange are given in Table 4. The data presented showed that in most of the treatments the readings for Key lime were higher than those of either Rough lemon or sour orange. The figures with respect to Rough lemon and sour orange were highly variable. However, the resistance readings of seedlings of all the three species showed an almost consistent trend (Figs. 6, 7, 8). This was evident in that the seedlings exposed to low temperatures had lower resistance readings than the unhardened seedlings. In the case of seedlings exposed to freezing temperatures, the unhardened seedlings had higher readings than those of the hardened seedlings. Also the resistance readings for the unhardened frozen seedlings were reduced considerably as compared to the readings for the unhardened seedlings before they were exposed to freezing temperatures.

TABLE 4

THE AVERAGE RESISTANCE READINGS*, EXPRESSED IN OHMS (x 10^3), OF THE INTACT MAIN AXES OF SEEDLINGS OF KEY LIME, ROUGH LEMON AND SOUR ORANGE

Kind of seedlings	Before freezing						After freezing					
	Unhardened			Hardened			Unhardened			Hardened		
	T	M	B	T	M	B	T	M	B	T	M	B
Key lime	134.4	85.0	61.8	126.9	63.1	55.0	91.5	60.6	49.9	64.3	28.9	27.7
Rough lemon	102.4	43.0	40.0	67.5	51.1	55.7	53.4	29.0	33.1	41.7	38.1	29.7
Sour orange	98.7	54.1	49.9	78.7	55.3	49.9	80.0	41.2	42.5	51.4	27.9	27.7

*These readings were taken by a probe with needles 1 cm. apart. Each entry in this table is the average of eight seedling.

T = Top of seedling
M = Middle of seedling
B = Base, lower part of seedling

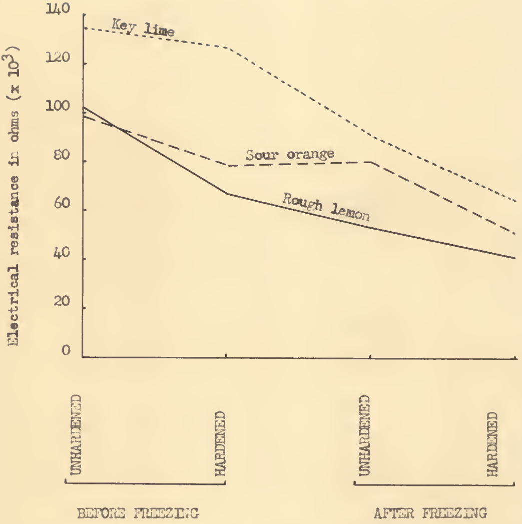


Fig. 6. Electrical resistance readings in ohms (x 10^3), taken near the second node from the apex of the main axes of seedlings of Key lime, Rough lemon and sour orange.

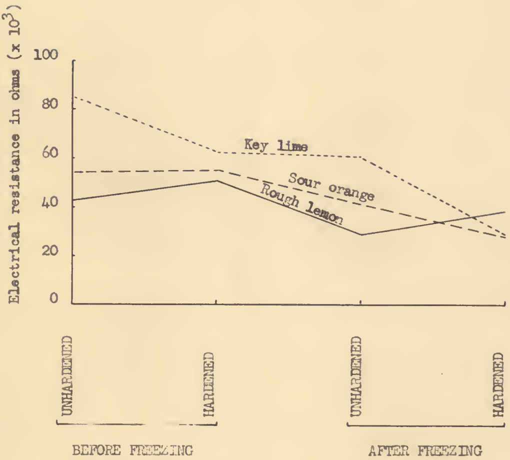


Fig. 7. Electrical resistance readings in ohms (x 10^3), taken at the middle of the main axes of seedlings of Key lime, Rough lemon and sour orange.

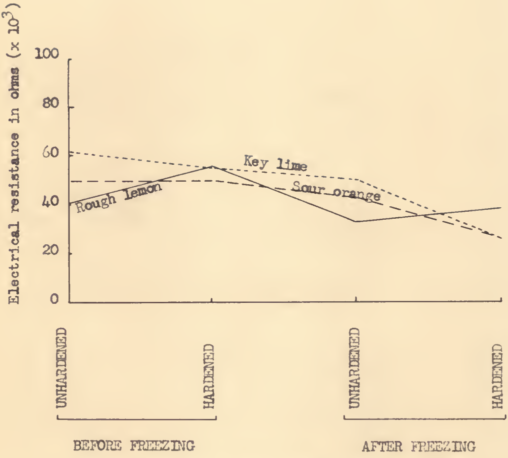


Fig. 8. Electrical resistance readings, ohms (x 10^3), taken near the base of the seedlings of Key lime, Rough lemon and sour orange.

In the above experiments the seedlings used for taking electrolytic readings were different for different temperature treatments. It was considered worthwhile to investigate the response of some additional seedlings with respect to the electrical resistance when the same groups of seedlings were given the different temperature treatments. For this experiment, ten seedlings each of Rough lemon and sour orange were randomly selected and placed in the growth chamber at 78°F. After two weeks electrical resistance readings were taken at three points on each seedling in the manner described for taking readings on intact seedlings (page 25). The seedlings were then placed in the cold chamber at 75°F. and the temperature lowered to 35°F. at the rate of 10°F. per three hours. After the seedlings were maintained at 35°F. for three hours, the temperature in the cold chamber was lowered to 18°F. at the rate of 2°F. per hour. The seedlings were maintained at 18°F. for three hours and then brought back to 35°F. at the rate of 2°F. per hour. After the seedlings had thawed, they were placed in the growth chamber and electrical resistance readings were taken. These readings were taken at a point 90° around the stem portion from where the readings were taken before the seedlings were subjected to cold temperature. The seedlings were allowed to stay in the growth chamber (78°F.) for three weeks after which their recovery data were taken.

The results of this experiment are given in Table 5. The results are expressed in percent decrease in the electrical readings of the seedlings exposed to freezing temperature over the seedlings before they were frozen.

TABLE 5

PERCENT DECREASE IN RESISTANCE READINGS OF SEEDLINGS OF ROUGH LEMON AND SOUR ORANGE AFTER THEY WERE SUBJECTED TO FREEZING TEMPERATURE AND THE PERCENT RECOVERY OF THE CORRESPONDING SEEDLINGS

Kind and number of seedling	Percent decrease in electrical readings			Percent recovery
	Top	Middle	Bottom	
Rough lemon				
1	67.5	63.6	92.1	25
2	64.8	72.2	92.5	25
3	51.7	64.6	75.5	25
4	69.7	76.5	86.9	0
5	55.3	83.3	88.7	25
6	68.1	69.0	91.8	25
7	63.5	78.4	88.7	0
8	57.3	75.0	91.2	0
9	60.0	70.9	88.3	0
10	60.0	78.8	89.6	25
Sour orange				
1	62.5	79.1	92.3	0
2	56.2	58.1	94.2	0
3	64.0	52.0	93.5	0
4	54.5	63.4	95.0	0
5	57.6	31.8	93.0	0
6	66.6	62.5	93.0	0
7	54.5	68.2	91.6	0
8	76.9	72.5	93.7	0
9	66.6	63.1	91.2	0
10	78.3	83.3	90.0	0

The statistical analysis of the data presented in Table 5 showed that the resistance readings taken on the intact plant tissue had no correlation with the growth-chamber recovery of the seedlings. The correlation coefficients calculated for finding relationship between the resistance readings and their recovery indexes were -0.14 for the top tissue, +0.199 for the middle and -0.127 for the bottom tissue of the seedlings. None of these 'r' values was statistically significant.

C. Estimation of Water-soluble Proteins

1. First series of experiments

Group B1 comprising nine seedlings each of Key lime, Rough lemon and sour orange was maintained at 78°F. for four weeks. These seedlings were then analysed chemically for water-soluble proteins. Group D1 having the same number of seedlings of the three species of citrus was hardened at 38°F. for two weeks, after which the soluble protein content of the seedlings was determined. The results of these experiments are given in Table 6.

2. Second series of experiments

Eight seedlings each of Key lime, Rough lemon and sour orange comprising group B2 were grown at 78°F. for four weeks. These seedlings were then analysed chemically for water-soluble proteins. Group F2 having the same number of seedlings of the three species of citrus was maintained at 38°F. for ten days. Following this hardening treatment, the seedlings were analysed chemically for water-soluble proteins. The data with respect to the quantitative analyses made during these experiments are presented in Table 7.

TABLE 6

PERCENT WATER-SOLUBLE PROTEINS IN THE LEAF AND BARK TISSUE OF KEY LIME, ROUGH LEMON AND SOUR ORANGE SEEDLINGS, USED IN THE FIRST SERIES OF EXPERIMENTS

Kind of seedlings	Percent water-soluble proteins*	
	Unhardened seedlings	Hardened seedlings
Key lime	4.637	5.973
Rough lemon	5.584	3.523
Sour orange	4.749	2.848

*Mean of nine seedlings of each species.

TABLE 7

PERCENT WATER-SOLUBLE PROTEINS IN THE LEAF AND BARK TISSUE OF KEY LIME, ROUGH LEMON AND SOUR ORANGE SEEDLINGS, USED IN THE SECOND SERIES OF EXPERIMENTS

Kind of seedlings	Percent water-soluble proteins*	
	Unhardened seedlings	Hardened seedlings
Key lime	4.663	5.961
Rough lemon	5.491	3.638
Sour orange	4.737	3.493

*Mean of eight seedlings of each species.

The results of the first and the second series of experiments, summarized in Tables 6 and 7 respectively, showed that in the unhardened seedlings Rough lemon had the highest water-soluble proteins. The sour orange seedlings were intermediate in their soluble protein content and the Key lime had the least amount of the three species sampled. On the other hand, when the hardened seedlings were analysed, Key lime showed the highest amount of water-soluble proteins while Rough lemon and sour orange followed next in that order. The data also showed that the Key lime seedlings increased in their soluble-protein content when they were hardened as compared to those in an unhardened condition. Rough lemon and sour orange seedlings, on the other hand, showed significant decreases in their protein content in the hardened state. In the first series of experiments when the seedlings were exposed to 38°F. for two weeks, the Key lime showed 28.8 percent increase in soluble protein over the unhardened seedlings. When the seedlings were hardened, Rough lemon and sour orange showed decreases of 58.5 and 66.7 percent respectively. In the second series of experiments, soluble proteins increased 27.8 percent in the Key lime, and decreased 50.9 and 35.6 percent, respectively, in Rough lemon and sour orange as a result of low temperature treatments.

D. Plasmolysis Test

The subsections made of the large sections of the bark tissue were stained with neutral red after they had been plasmolyzed and deplasmolyzed. The stained subsections were then examined under the microscope. This examination revealed great variations in the range of mortality of cells as estimated by the staining after plasmolysis.

Sections obtained from the same seedling also varied considerably as to their intensity of staining. Similar variations were noted in the sections obtained from the bark of Key lime, Rough lemon and sour orange.

In the plasmolysis test, the mortality rate of the cells could not be ascertained quantitatively. It was noted, however, that the sections were comparatively more intensely stained as the concentration of the plasmolyzing solution was reduced from 5M to 2.5M or 1M. This difference could not be expressed quantitatively to form any basis for estimating the relative cold hardiness of the citrus seedlings of different species.

E. Actual Freezing Test

1. First series of experiments

After exposing them to 23°F. for three hours, the hardened seedlings of group F1 were allowed to thaw at 35°F. and were then placed in the growth chamber at 78°F. for three weeks. At that time they were scored for their recovery. The recovery indexes and the total percent recovery as computed by the recovery formula are given in Table 8.

The figures in Table 8, using the rating system explained on page 30, showed that the Key lime seedlings had the least percent recovery of all the three species which were exposed to the freezing temperature. Sour orange, on the other hand, showed the highest percent recovery (58.33). The Rough lemon seedlings were intermediate in their cold hardiness with 52.77 percent recovery. Although Rough lemon and sour orange seedlings were not statistically different

TABLE 8

GROWTH-CHAMBER RECOVERY OF HARDENED SEEDLINGS OF THREE SPECIES OF CITRUS, THREE WEEKS AFTER THEY WERE FROZEN AT 23°F. FOR THREE HOURS

Seedling number	Ratings*		
	Key lime	Rough lemon	Sour orange
1	0	3	4
2	0	1	4
3	0	2	3
4	4	2	2
5	0	4	2
6	0	2	1
7	0	4	1
8	0	1	1
9	0	0	3

	Total percent recovery
Key lime	11.11
Rough lemon	52.77
Sour orange	58.33

*See page 30 for explanation.

in their respective cold hardiness ratings, both species were significantly hardier than Key lime, which showed only 11.11 percent recovery.

2. Second series of experiments

After subjecting them to their respective temperature treatments, the seedlings comprising groups D2 and H2 were exposed to 23°F. for three hours and then were allowed to thaw at 35°F. When the seedlings had thawed, they were placed in the growth chamber at 78°F. for three weeks. The seedlings were then scored for their recovery. These indexes, and the data as computed by the recovery ratings and the percent recovery formula, are given in Table 9.

The statistical analysis of the data regarding the actual freezing tests of the seedlings (Table 9) showed that the differences in the degree of recovery of seedlings when they were exposed to the freezing temperatures without hardening and after hardening were highly significant. As to the relative cold hardiness of the three species under study, no significant differences were shown. A trend, however, was clearly noticeable from the data presented in Table 9. When the seedlings were frozen after hardening, Key lime showed a very low recovery rate. The Rough lemon and sour orange seedlings, on the other hand, showed much higher rates of recovery and both reacted in a relatively similar manner. When the seedlings were exposed to the freezing temperature in the unhardened state, sour orange showed the highest recovery and the Key lime seedlings had the least percent recovery for the comparable treatment. The statistical

TABLE 9

GROWTH-CHAMBER RECOVERY OF SEEDLINGS OF KEY LIME (KL), ROUGH LEMON (RL), AND SOUR ORANGE (SO), THREE WEEKS AFTER THEY WERE FROZEN AT 23°F. FOR THREE HOURS

Seedling number	Ratings					
	Unhardened seedlings			Hardened seedlings		
	KL	RL	SO	KL	RL	SO
1	1	3	1	0	1	2
2	1	3	4	0	2	3
3	1	4	3	0	0	0
4	4	2	3	0	0	2
5	4	1	3	0	2	2
6	4	2	4	0	4	4
7	4	2	2	0	2	0
8	1	4	3	1	1	0

	Total percent recovery	
	Unhardened seedlings	Hardened seedlings
Key lime	62.50	3.12
Rough lemon	65.62	37.50
Sour orange	71.87	40.62

(Table 9, continued)

Analysis of variance

Source of variance	d.f.	Sum of squares	Mean Square
Temp. Treatments	1	30.083	30.083 **
Species	2	7.875	3.937
RL vs SO	1	0.281	0.281
RL and SO vs KL	1	7.594	7.594 *
Treatment x RL vs SO	1	0.021	0.021
Treatment x RL and SO vs KL	1	3.760	3.760
Error	42	60.500	1.440
Total	47		

*Significant at 5 percent level.
**Significant at 1 percent level.

analysis of the data showed that while there was no significant difference in the degree of cold hardiness of Rough lemon and sour orange, there was significant difference between Rough lemon and sour orange taken together and Key lime. The data further showed that the seedlings which were exposed to low temperature (38°F.) had significantly lower percent recovery than the seedlings which were not exposed to 38°F. before freezing.

This fact is also evident from the Figs. 10 and 12 which are of the same seedlings as Figs. 9 and 11 respectively. The seedlings shown in Fig. 9 were exposed to the freezing temperature without hardening and those in Fig. 11 were maintained at 38°F. for ten days before they were treated with 23°F. The unhardened seedlings received less injury due to cold (Fig. 10) than the seedlings which had been exposed to 38°F. for ten days prior to cold treatment (Fig. 12).

These data showed the necessity of investigating a proper hardening temperature which could induce cold tolerance in the citrus seedlings. For this, comparable seedlings as used in the earlier experimentation were randomly selected and grown in the growth chamber at 78°F. for four weeks. After this the seedlings were subjected to the following treatments.

1. Sixteen-hour day at 60°F. constant day and night.
2. Eight-hour day at 60°F. constant day and night.
3. Sixteen-hour day at 60°F. for day and 38°F. at night.
4. Eight-hour day at 60°F. for day and 38°F. at night.
5. Sixteen-hour day at 38°F. constant day and night.
6. Eight-hour day at 38°F. constant day and night.

Figure 9. (L to R) Rough lemon, Key lime and sour orange seedlings grown at 78°F. for four weeks.

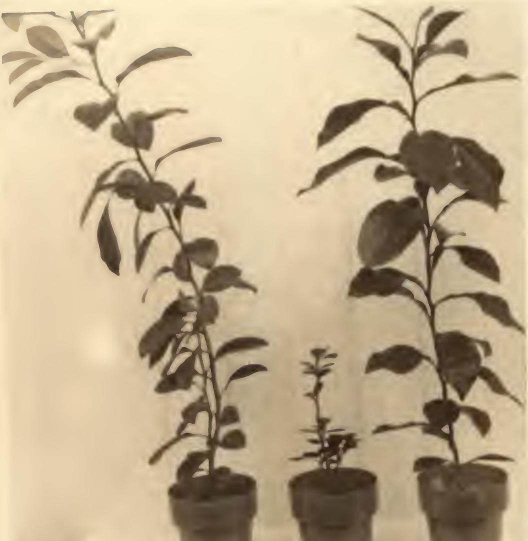
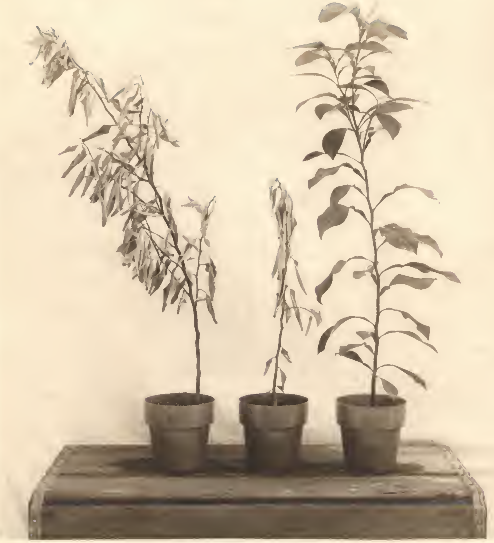


Figure 10. (L to R) Rough lemon, Key lime and sour orange seedlings three weeks after they were exposed to 23°F. for three hours.

Figure 11. (L to R) Rough lemon, Key lime and sour orange seedlings exposed to 38°F. for ten days.

Figure 12. (L to R) Rough lemon, Key lime and sour orange seedlings three weeks after they were exposed to 23°F. for three hours.



In each treatment there were seven seedlings each of Rough lemon and sour orange. When the seedlings had received the respective temperature treatments outlined above, they were exposed to 18°F. for three hours in the manner previously described. Following the cold treatment, the seedlings were placed in the growth chamber with a 14-hour day and constant temperature of 78°F. They remained under these conditions for three weeks before they were scored for their recovery.

The results of this experiment are presented in Table 10. The statistical analysis of the data showed that the low constant temperature (38°F.) was significantly inferior in hardening effect to the high constant temperature (60°F.). For the latter temperature treatments the percent recovery was 42.85 and 7.14 for sour orange and Rough lemon respectively under 16-hour day and 42.85 each for sour orange and Rough lemon under 8-hour day. Under low constant temperature, on the other hand, the percent recovery was 3.57 for sour orange and 25.0 for Rough lemon under 16-hour day and 14.28 and 17.85 respectively for the two species under the 8-hour day. The results further showed that the high-day (60°F.) and low-night (38°F.) temperature was significantly superior to either high or low constant temperatures.

When the seedlings were treated with either high or low constant temperatures, the effect of day length on the induction of cold hardiness was not significant. Nevertheless, under the high-day and low-night temperature conditions, the effect of day length on the hardening of citrus seedlings was highly significant in favor of the 16-hour day.

TABLE 10

THE EFFECT OF TEMPERATURE AND DAY LENGTH ON THE COLD HARDENING OF ROUGH LEMON (RL) AND SOUR ORANGE (SO)

Recovery scores											
60°F. constant				38°F. constant				60°F. day, 38°F. night			
16-hour day		8-hour day		16-hour day		8-hour day		16-hour day		8-hour day	
RL	SO	RL	SO	RL	SO	RL	SO	RL	SO	RL	SO
0	2	2	2	0	0	0	0	3	4	0	2
0	2	1	2	1	0	0	1	2	3	1	2
0	2	2	1	0	1	0	1	2	3	1	0
0	2	2	0	1	0	1	1	1	3	1	2
1	1	2	4	1	0	0	0	3	1	2	1
0	1	2	1	0	0	4	1	3	1	1	1
1	2	1	2	4	0	0	0	2	1	1	1
Percent recovery											
7.14	42.85	42.85	42.85	25.0	3.57	17.85	14.28	57.14	57.14	25.0	35.71

(Table 10, continued)

STATISTICAL ANALYSIS OF THE DATA TAKING THE DEGREE OF COLD INJURY INTO CONSIDERATION, USING INDEXES OF SEEDLINGS WHICH WERE COMPLETELY DEAD (0) OR RECEIVED SEVERE INJURY (1)

Source of variance	d.f.	Sum of squares	Chi square
Temperature effect for full day	1	2.5714	11.200 **
Period of light, same temperature for full day	1	0.2857	1.244 N.S.
Interaction	1	0.2857	1.244 N.S.
Period of light for same temperature difference day and night	1	1.2857	5.600 *
Same temperature all day vs high-day and low-night temperature	1	0.8571	3.733 N.S.

STATISTICAL ANALYSIS OF THE DATA TAKING INTO CONSIDERATION THE DEGREE OF RECOVERY OF THE SEEDLINGS, USING INDEXES OF SEEDLINGS WHICH WERE COMPLETELY RECOVERED (3) OR RECEIVED NO INJURY (4)

Source of variance	d.f.	Sum of squares	Chi square
Temperature effect for a full day	1	0.01785	0.1678 N.S.
Period of light, same temperature for full day	1	0.01785	0.1678 N.S.
Interaction	1	0.01785	0.1678 N.S.
Period of light for same temperature difference day and night	1	1.75	17.50 **
Same temperature all day vs high-day and low-night temperature	1	0.7202	9.40 *

N.S. = Not significant
* = Significant at 5 percent level
** = Significant at 1 percent level

The percent recovery under the high-day and low-night temperature conditions was 57.14 each for sour orange and Rough lemon for 16-hour day and 35.71 and 25.0, respectively, for the two species under the 8-hour day conditions.

Another experiment was conducted to investigate further the favorable hardening conditions for citrus. Seventy-seven sour orange seedlings were grown in the growth chamber at 78°F. for four weeks. These were then divided into 11 equal groups and subjected to the following treatments.

1. Illuminated 24 hours continuously
    a. Sixteen hours at 78°F., eight hours at 50°F.
    b. Sixteen hours at 60°F., eight hours at 50°F.
2. Illuminated 16 hours, in darkness eight hours
    a. Sixteen hours at 78°F., eight hours at 50°F.
    b. Sixteen hours at 78°F., eight hours at 44°F.
    c. Sixteen hours at 78°F., eight hours at 38°F.
    d. Sixteen hours at 78°F., eight hours at 32°F.
    e. Sixteen hours at 60°F., eight hours at 50°F.
    f. Sixteen hours at 60°F., eight hours at 44°F.
    g. Sixteen hours at 60°F., eight hours at 38°F.
    h. Sixteen hours at 60°F., eight hours at 32°F.
    i. Sixteen hours at 60°F., eight hours at 38°F. and immediately after this 100°F. for ten minutes.

After each of the groups had received the respective treatment for ten days, the seedlings were exposed to 18°F. for three hours and subsequently placed in the growth chamber in the manner previously described. After three weeks the seedlings were scored for their recovery. The results of this experiment are summarized in Table 11.

The statistical analysis of the data presented in Table 11 showed that the effect of low-night temperatures on the induction of hardiness was highly significant. There was a direct linear relationship between the low-night temperatures and the rates of recovery of the seedlings; 32°F. night temperature gave the highest recovery followed by 38°F., 44°F. and 50°F. in that order (Fig. 13). The day temperature had a significant influence on the cold hardiness but this effect was dependent on the prevailing night temperatures.

In the case of seedlings which were exposed to 100°F. for ten minutes every day after the 38°F. treatment, no significant difference was found in their degree of cold hardiness as compared to the seedlings of other treatments. The former seedlings, however, had lower percent recovery as compared to the 2g treatment where the seedlings were not exposed to 100°F. after the low-temperature treatment.

Under the conditions of this experiment, the 24-hour illumination period was much superior to 16-hour illumination period. This effect was independent of the day temperature to which the seedlings had been exposed.

TABLE 11

THE EFFECT OF TEMPERATURE AND DAY LENGTH ON THE COLD HARDENING OF SOUR ORANGE SEEDLINGS

	Recovery Scores										
Illumination period	24-hour		16-hour								
Day temperature	78°F.	60°F.	78°F.				60°F.				
Night temperature	50°F.	50°F.	50°F.	44°F.	38°F.	32°F.	50°F.	44°F.	38°F.	32°F.	38°F. and 100°F. for 10 minutes
	1	2	0	1	1	2	2	1	1	1	1
	1	2	0	1	1	2	1	1	1	1	1
	1	2	1	1	1	2	1	2	2	1	1
	1	2	0	1	1	2	1	2	2	2	1
	1	2	1	1	2	1	2	1	2	2	1
	1	2	0	1	2	2	1	1	2	2	1
	0	1	1	1	2	4	1	2	1	3	1
	Percent recovery										
	21.43	46.43	10.71	25.00	35.71	53.57	32.14	35.71	39.28	42.85	25.00

(Table 11, continued)

Analysis of variance			
Source of variance	d.f.	Sum of squares	Mean Square
A. Night temperature effect			
Linear N1	1	3.571	3.571 **
Quadratic N2	1	0.047	0.047 N.S.
78°F. vs 60°F. T	1	0.023	0.023 N.S.
T x N1	1	1.285	1.285 *
T x N2	1	0.047	0.047 N.S.
B. Illumination period			
24-hour vs 16-hour	1	1.750	1.750 *
78 F. vs 60 F.	1	6.035	6.035 **
Interaction	1	0.035	0.035 N.S.
A vs B	1	3.259	3.259 **
10 minutes at 100 F. vs all others			
	1	0.877	0.877 N.S.

N.S. = Not significant.
* = Significant at 5 percent level.
** = Significant at 1 percent level.

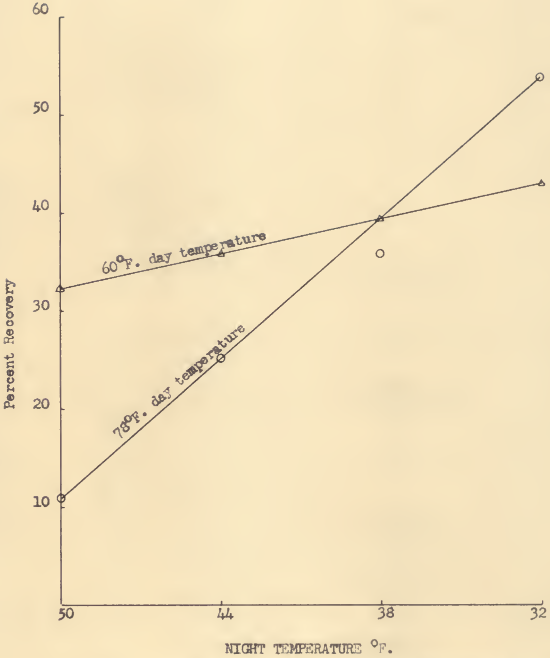


Fig. 13. The effect of different low-night temperatures on the cold hardiness of sour orange seedlings, under the 78°F. and 60°F. day-temperature conditions.

V. DISCUSSION AND CONCLUSIONS

A. Electrical Conductivity of Tissue Extract

The electrical conductivity method of measuring the injury from low temperature as suggested by Dexter and co-workers (16, 17) did not show any promise during these studies. Key lime, which is considered to be less cold hardy than either Rough lemon or sour orange (83), had the highest electrical resistance readings (supposedly indicative of highest cold hardiness) when frozen in an unhardened condition (Tables 1 and 2). When the seedlings were exposed to the freezing temperatures in a hardened condition, the resistance readings for Key lime were also higher than those for Rough lemon and sour orange (Table 1). Similarly, the electrical resistance readings of the seedlings of the three species taken before they were subjected to hardening temperature (38°F.) and after hardening of seedlings failed to show any correlation with their respective cold hardiness.

The percent decrease in the resistance readings for hardened and frozen seedlings over those for the unhardened seedlings (Fig. 5) indicated that the Key lime seedlings should be the most cold hardy of the three species of citrus. This is directly opposite to their capacity of survival in the field (83).

These data, therefore, indicate that the electrical resistance readings do not show the order of cold hardiness of the three species studied. This was further confirmed by the results of the actual freezing tests (Tables 8 and 9), whereby the Key lime seedlings showed the least degree of recovery after they were exposed to freezing temperatures. Sour orange was found to be the most cold hardy of the three species. The resistance readings, on the contrary, failed to show this pattern in signifying the relative cold hardiness of the seedlings.

Many workers have determined the cold hardiness of plants with the use of the electrical conductivity method. Most of them, however, used this method to measure the cold hardiness of varieties within a species (6, 12, 23, 26, 62). Dexter (15) in one of his comparatively recent publications stated that "in the original work with the method, where the hardiness of varieties of apples, alfalfa, clover, small grains, raspberries, etc., was compared, no method seemed fully suitable in comparing species, even when total electrolytes were taken into consideration, since contents of dry matter, electrolytes, etc., varied so greatly, and since the speed of exosmosis was not comparable."

The results of the present studies are in agreement with conclusions of Dexter (15). The electrical conductivity method, based on the exosmosis of electrolytes from the injured cells, does not seem to be satisfactory for measuring the relative cold hardiness of different species of citrus.

B. Electrical Conductivity of Intact Tissue

The electrical resistance readings taken on the intact seedlings (Tables 3 and 4) did not show any correlation with cold hardiness. Although the data showed that the electrical readings decreased considerably after the seedlings were exposed to freezing temperatures as compared to the pre-freezing treatments (Figs. 6, 7 and 8), there was no consistent pattern in the readings to form any basis for valid comparisons among the species of citrus.

The experiment in which the same seedlings were used for all the temperature treatments and the results were expressed in percent decrease in the resistance readings of the frozen seedlings over the readings for the seedlings which were not subjected to the freezing temperature, also failed to show any correlation between the electrical resistance and the degree of recovery of the respective seedlings (Table 5).

It seems that the same inherent drawbacks, such as the differences in the contents of dry matter and electrolytes and the degree of release of electrolytes from the plant cells, influence the tissue extract and the intact-plant modifications of the electrical conductivity method. Under the conditions of the present experiments it seems safe to conclude that the electrical conductivity method, using tissue extract as well as the intact plant, can not be used to measure quantitatively the relative cold hardiness of various species of citrus.

C. Estimation of Water-soluble Proteins

In their work on the black locust, Briggs and Siminovitch (4, 69, 71) found a direct correlation between the amount of water-soluble proteins and the cold hardiness of the plants sampled. On the basis of the analytical studies they (69) concluded that the seasonal variations in the cold hardiness of the bark tissue were closely proportional to the simultaneous variations in the soluble protein content of the cells. During the present studies, in a marked contrast to the above observation, no such correlation could be found between the cold hardiness of citrus seedlings and their soluble protein content. The hardened Key lime seedlings (Table 6) showed an average of 5.973 percent water-soluble proteins, higher than both Rough lemon (3.523 percent) and sour orange (2.848 percent). The data obtained during the second series of experiments gave comparable results. The actual freezing tests (Tables 8 and 9), on the other hand, showed that the Key lime seedlings were the most tender of the three species under study. The Rough lemon seedlings which had been subjected to 38°F. for the hardening treatment also had more soluble proteins than the sour orange seedlings although the former were shown to be less cold hardy (Table 9). The data with respect to the soluble protein content of the seedlings which were not exposed to 38°F. also failed to show any correlation with the recovery data of the seedlings of the three species of citrus.

Sakai (63) working with mulberry found that in September and in early May, at which times the artificial hardening was remarkably effective, the increase in the frost hardiness was closely proportional to the increase of the sucrose concentration, but not to that of the water-soluble protein.

The biochemical studies on the living plant cells have shown that the water-soluble proteins increase and decrease with the corresponding change in the cold hardiness of the tissue (4, 69, 71). As the first step in the hardening process in a cell, the amount of water-soluble proteins increase in the protoplasm. But this increase in proteins is not the only chemically determinable change on hardiness (73).

Siminovitch and Chater (73) observed a remarkable increase in the extractibility of phospholipids during the hardening process and considered it probable that the resistance to cold injury may occur due to an increase in phospholipids independent of protein synthesis.

The data collected during the present studies support the findings of Siminovitch and Chater (73). The soluble protein content (Tables 6 and 7) of the seedlings did not follow the corresponding changes in their cold hardiness, and therefore can not be used as a satisfactory index of cold hardiness of citrus species.

D. Plasmolysis Test

Theoretically plasmolysis and deplasmolysis of plant tissue simulates to a considerable degree the freezing and thawing processes. The cold-hardy plants are better able to withstand the harmful effects of dehydration, whether it is due to extracellular ice formation in the frozen plant or to osmotic water removal, than the cold-susceptible plants. Considering this and some other advantages such as saving of time and cost of equipment, an attempt was made to standardize the plasmolysis test for measuring the cold hardiness of citrus seedlings.

In spite of a very careful control of uniformity of tissue sections and the timing of various operations in the test, the plasmolysis test failed to yield reliable data to form the basis for a quantitative measure of the cold hardiness of citrus seedlings. The absence of such a reliable basis was also reported by Dexter (15) whose tests for differential varietal desiccation injury did not show any promise in relation to winter hardiness evaluation of alfalfa. Sakai (63) working with mulberry reported that employment of the values of the dehydration resistance of the cells obtained by the Siminovitch method as a measure of the cold-resistance capacity involved risk.

The bark tissue to be used for the plasmolysis test should be mature and uniform to give reliable and reproducible results. In the case of eight-month-old citrus seedlings used in the present study, the bark tissue, even from the region of cortex and last season's phloem, was apparently not uniform. This might have caused the variability in the mortality rates of cells of different sub-sections made from one large section of the same seedling. Neutral red, however, gave good contrast between the areas where the stain had leached out and the areas where it had not leached out. Such a difference, however, could not, insofar as estimation of cold hardiness is concerned, be assessed on a quantitative basis.

E. Actual Freezing Test

Subjecting the seedlings to the freezing temperatures was found to be the most reliable quantitative method for measuring their cold hardiness. The recovery values obtained by this test agree favorably with the generally accepted degree of cold hardiness of each of the citrus species under study (83). In this test, the Key lime was found to be the least cold hardy, Rough lemon was medium in cold hardiness and sour orange was the most cold hardy of the three species.

Many workers have reported that the plants should be hardened at low, but above freezing, temperatures (1, 33, 76). The results of the actual freezing tests conducted in the present studies, on the other hand, showed that the low, near freezing, temperatures actually reduced the degree of cold hardiness of the citrus seedlings (Tables 9 and 10). Even the high constant temperature of 60°F. was significantly better than the low temperature of 38°F. for the induction of cold hardiness. The results of the hardening experiment (Table 10) showed that high-day and low-night temperature was best for inducting cold hardiness. These experiments further showed that the lower the night temperature, the greater was the cold hardiness induced in the citrus seedlings. This is in complete agreement with the findings of Ivanov (33).

The length of day had no measurable effect on the cold hardiness in either high or low constant-temperature treatments. In the case of seedlings which were exposed to high-day and low-night temperatures, the longer illumination period was significantly superior to the shorter illumination period (Tables 10 and 11). Under the conditions of these experiments it could not be determined whether the length of day (the illumination period) had a photoperiodic effect or whether the effect was simply due to the longer period at high temperature. Nevertheless, these results suggested that the effect of longer day length on the induction of cold hardiness was photosynthetic. This hypothesis is supported by the fact that under similar temperature conditions, the longer period of illumination was significantly superior in inducing cold hardiness than the shorter period. It seems that under the long-day conditions, the plants were able to accumulate a greater amount of photosynthetic materials than the plants under the short-day conditions. These materials in the form of soluble sugars are known to induce cold hardiness in plants (39, 48).

To conduct an actual freezing test, adequate precautions must be taken to assure the use of uniform seedlings grown under standard conditions of light, temperature, humidity and nutrition. The seedlings may be hardened at an alternating high-day and low-night temperature. This treatment should be given to the seedlings for at least ten days before subjecting them to freezing temperatures. The temperature should be lowered and raised at a standard rate. The post-thawing conditions should also be uniform from one test to the other to assure comparative values.

Actual freezing tests alone should be required where the objective of the hardiness testing, as in the present study, is a prediction of the capacity of a plant to survive when exposed to freezing conditions in the field (70). These tests provide a measure of cold hardiness for every part of the plant. Localized injury and other damages caused to meristematic tissues are very easily assessed by this method.

This method has another very important inherent advantage in that large numbers of citrus seedlings of various species can very easily be tested for their relative cold hardiness. The surviving seedlings can then be grown and used for further propagation of hardy varieties. This fact is of special importance in the case of small citrus seedlings, because in other tests such as estimation of soluble proteins or the electrical conductivity method, the seedlings are used up in conducting the tests and very little is left for further propagation if certain seedlings may prove to be hardier than others.

VI. SUMMARY

The need for a reliable quantitative method for measuring the cold hardiness of citrus seedlings is extremely important in any program designed to obtain cold-hardy citrus plants. The present study was started in 1957 at the University of Florida, Gainesville, with a view to evaluate the methods for measuring the cold hardiness of young citrus seedlings on a quantitative basis.

The electrical conductivity method, either with tissue extract or with intact-tissue, failed to show any correlation between the electrolytic readings and the degree of cold hardiness of the seedlings. Similarly, the chemical analysis of the leaves and bark tissue for water-soluble proteins and a plasmolysis test employing balanced salt solutions were found to be unsatisfactory as means of measuring the cold hardiness of young citrus seedlings of different species, or of the same species under different environmental conditions.

The actual freezing test was found to be the most reliable method for a quantitative measure of the cold hardiness. The climatic and cultural conditions for growing and testing of the young citrus seedlings for their cold hardiness have been presented. A method to be used in conjunction with the actual freezing test has been devised to estimate the relative cold hardiness of seedlings.

The actual freezing tests showed that the constant low, above-freezing, temperature reduced the cold hardiness of the citrus seedlings. A high day-temperature (78°F.) alternated with a low night-temperature

(32°F.) was better in inducing cold hardiness to citrus seedlings than either high or low constant temperature. These tests further showed that under the high day and low night temperature conditions, the longer the illumination period, the greater was the degree of cold hardiness induced in the seedlings.

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BIOGRAPHICAL SKETCH

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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